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Microbial Studies Supporting Implementation Of In Situ Bioremediation At Test Area North

Joni M. Barnes
Roger L. Ely
Gretchen Matthern
Catherine Rae

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Approved by:

Joseph S. Rothermel OU 1-07B Project Manager BBWI	Date

ABSTRACT

The Idaho National Engineering and Environmental Laboratory is evaluating in situ bioremediation of contaminated groundwater at its Test Area North Facility. To determine feasibility, microcosm and bioreactor studies were conducted to ascertain the ability of indigenous microbes to convert trichloroethene and dichloroethene to non-hazardous byproducts under aerobic and anaerobic conditions, and to measure the kinetics of microbial reactions associated with the degradation process.

Microcosms were established from core samples and groundwater obtained from within the contaminant plume. These microcosms were amended with nutrients, under aerobic and anaerobic conditions, to identify electron donors capable of stimulating the degradation process. Results of the anaerobic microcosm studies showed that lactate, acetate and propionate amendments stimulated indigenous cell growth and functioned as effective substrates for reductive degradation of chloroethenes. Bioreactors inoculated with cultures from these anaerobic microcosms were operated under a batch mode for 42 days then converted to a fed-batch mode and operated at a 53-day hydraulic residence time.

It was demonstrated that indigenous microbes capable of complete anaerobic reductive dechlorination are present in the subject well. It was also demonstrated that aerobic microbes capable of oxidizing chlorinated compounds produced by anaerobic reductive dechlorination are present. Kinetic data suggest that controlling the type and concentration of electron donors can increase trichloroethene conversion rates. In the event that complete mineralization of trichloroethene does not occur following stimulation, an anaerobic/aerobic treatment scheme is feasible.

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FIGURES

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ACRONYMS

ARD	anaerobic reductive dechlorination
DCE	dichloroethene
FID	flame ionization detector
GC	gas chromatograph
HP	Hewlett Packard
HRT	hydraulic residence time
IC	intrinsic control
ID	inside diameter
INEEL	Idaho National Engineering and Environmental Laboratory
KC	killed control
SPME	Solid Phase Microextraction
SSR	sum of squared residuals
TAN	Test Area North
TCE	trichloroethene
TCD	thermal conductivity detector
VC	vinyl chloride

Microbial Studies Supporting Implementation of In Situ Bioremediation at Test Area North

1. INTRODUCTION

Biodegradation is being considered as an *in situ* treatment option for remediation of contaminated groundwater at Test Area North (TAN) of the Idaho National Engineering and Environmental Laboratory (INEEL). The contaminant plume, which contains trichloroethene (TCE) and related daughter products, is associated with an injection well and waste storage ponds. The injection well was used between 1953 and 1972 to dispose of chlorinated solvents, organic sludges, sanitary sewage, and radioactive waste.

These studies, which were intended to assess microbial activity near the injection well, included two goals. The first goal was to determine the potential for indigenous microbes at TAN to completely convert TCE to non-hazardous end products under in situ conditions. The second goal was to measure the kinetics and stoichiometry of the microbial reactions associated with the degradation process.

Bioremediation at TAN most likely will involve anaerobic treatment at the hotspot to initiate TCE degradation followed by oxidation of daughter products in the aerobic portion of the plume. Consequently, experiments were conducted to evaluate the potential effects of two separate bioremediation strategies. The first scenario involves the complete conversion of TCE to ethene under anaerobic conditions via anaerobic reductive dechlorination (ARD). The second scenario relies on anaerobic conversion of TCE to dichloroethene (DCE) and vinyl chloride (VC) with subsequent aerobic biodegradation of the DCE and VC.

The experimental approach used in this work included microcosm and fed-batch reactor studies using aquifer materials obtained from Well TAN-37. The microcosm studies were conducted to assess the capabilities of indigenous microbes to degrade TCE under aerobic and anaerobic conditions and to identify electron donors capable of stimulating the degradation process. Fed-batch reactor studies were conducted to determine degradation kinetics, which in turn would be used in the design of the field evaluation plan.

2. METHODS

2.1 Chemicals

TCE and 1,2-*cis*-DCE were obtained from Sigma Chemical Company (St. Louis, MO). Certified standard solutions of lactate, acetate, propionate, butyrate, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, and VC were prepared by AccuStandard Inc. (New Haven, CT). Certified methane, ethane, and ethene standards were obtained from Scott Specialty Gases (Plumsteadville, PA). Certified anion solutions were obtained from Dionex (Sunnyvale, CA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

2.2 Analytical Techniques

2.2.1 Chloroethene Analysis

TCE, *cis*-DCE, *trans*-DCE and 1,1-DCE were analyzed using the Solid Phase Microextracton (SPME) technique (Arthur et al. 1992). The analytes were extracted from headspace gases for a period of 6 minutes using an 85-µm polyacrylate coated fiber (Supelco, Bellefonte, PA). Immediately following extraction, the analytes were desorbed in the injector of a Hewlett Packard (HP) Model 5890 Series II (Hewlett Packard, Palo Alto, CA) gas chromatograph (GC) onto a 30 m, 0.32 mm inside diameter (ID), 1.8 µm df, RTx-624 (Restek, Bellefonte, PA) chromatograph column using a splitless mode. The injector was fitted with a 1 mm SPME liner and maintained at 250°C. Helium was used as the carrier gas at a flow rate of approximately 2 mL/minute. During the assay, the column temperature was initially maintained at 60°C for 6.5 minutes and then increased to 180°C at a rate of 70°C per minute. Analytes were detected using a flame ionization detector (FID) maintained at 280°C. The GC was calibrated using in-house prepared standards. The calibration was verified prior to analysis using certified control samples. Utilizing this technique, the minimum detection limit for TCE, *cis*-DCE, *trans*-DCE, and 1,1-DCE, was 50 ppb. This technique was also used to measure VC; however, a 75 µm carboxen/polydimethylsiloxane coated fiber was used and the extraction time was increased to 10 minutes resulting in a lower detection limit of 5 ppb.

2.2.2 Ethane and Ethene Analysis

Headspace gas was analyzed for ethane and ethene by directly injecting a 100-µL sample into a HP Model 5890 Series II GC equipped with a FID and a 25 m, 0.32 mm ID PoraPLOT U column (Chrompack, Raritan, NJ). Helium was used as the carrier gas at a flow rate of 6.5 mL/minute. Throughout the assay, the column temperature was maintained at 35°C, the injector temperature at 225°C, and the detector temperature at 250°C. The GC was calibrated using certified gas standards, and control samples were assayed daily. Using this technique, the minimum detection limit for both ethane and ethene was 2 ppmv.

2.2.3 Methane and Oxygen Analysis

Headspace gas was analyzed for methane and oxygen by directly injecting a 100-µL sample into a HP Model 5890 Series II GC equipped with a thermal conductivity detector (TCD). Helium was used as the carrier gas at a flow rate of 5 mL/minute. The separation column was a 25 m, 0.53 mm ID silica PLOT Molsieve 5A (Chrompack, Raritan, NJ). Throughout the assay, the column temperature was maintained at 100°C, the injector temperature at 200°C, and the detector temperature at 225°C. The GC was calibrated using certified standards, and certified quality control standards were assayed daily. Using this technique, the minimum detection limit for methane was 100 ppmv and that of oxygen was 0.5%.

2.2.4 Acetate, Propionate, and Butyrate Analysis

Acetate, propionate, and butyrate levels were measured by filtering culture samples (0.2-µm pore size) and adjusting the pH to 2.0 using concentrated phosphoric acid. Typically, 200 µL of acid was added per 1.0 mL of filtrate and then 1 µL of the acidified solution was injected directly into a HP Model 5890 Series II GC equipped with a FID and a 30 m, 0.53 mm, 0.5 µm df, Nukol column (Supelco, Inc., Bellefonte, PA). Helium was used as the carrier gas at a flow rate of 8.2 mL/minute. Throughout the assay, the column temperature was maintained at 125°C, the injector temperature at 225°C, and detector temperature at 250°C. The GC was calibrated using certified standards, and control standards were assayed prior to running test samples. Using this technique, the minimum detection limit for acetate, propionate, and butyrate was 0.5 mg/L.

2.2.5 Methanol Analysis

Methanol concentration was analyzed by directly injecting 1 µL of filtered culture sample into a HP Model 5890 Series II GC equipped with a FID and a 30 m, 0.25 mm, 0.5 df, XTI-5 column (Restek, Bellefonte, PA). Helium was used as the carrier gas at a flow rate of 8.0 mL/minute. Throughout the assay, the column temperature was maintained at 60°C, the injector temperature at 100°C, and detector temperature at 125°C. The GC was calibrated using a certified standard, and control standards were assayed with each analytical run. Using this technique, the minimum detection limit for methanol was 0.25 mg/L.

2.2.6 Chloride, Sulfate, Nitrate, and Nitrite Analysis

Anion concentrations were measured in filtered culture samples using a Dionex 4000i (Dionex, Sunnyvale, CA) ion chromatograph instrument equipped with a conductivity detector and IonPac AG12A guard and analytical columns (Dionex, Sunnyvale, CA). A 2.7-mM sodium carbonate 0.3-mM sodium bicarbonate eluant was pumped through the columns at a flow rate of 1.5 mL/minute. A 20-mN sulfuric acid solution was used as the anion suppression regenerant. The instrument was calibrated using commercial reference standards, and certified quality control standards were assayed before sample analysis. Using this technique, the minimum detection limit for sulfate, nitrate, and nitrite was 0.5 mg/L and that of chloride was 0.15 mg/L.

2.2.7 Lactate Analysis

Lactate concentration was determined in filtered culture samples using a Dionex 4000i (Dionex, Sunnyvale, CA) ion chromatograph instrument equipped with a conductivity detector. The analytical column was an IonPac ICE-AS6 (Dionex, Sunnyvale, CA). A 0.4-mM nitric acid eluant was pumped through the column at a flow rate of 1.5 mL/minute. A 5.0-mM tetrabutylammonium hydroxide was used as the anion suppression regenerant. The instrument was calibrated using commercial reference standards, and certified quality control standards were assayed before sample analysis. Using this technique, the minimum detection limit for lactate was 2 mg/L.

2.3 Laboratory Microcosms

2.3.1 Matrix Materials

Aquifer core collected from Well TAN-37 and groundwater from Well TAN-29 were used in these studies. The core was obtained using sterilized equipment and was processed and sieved to a maximum size of 0.6 cm under an argon atmosphere. Groundwater was collected into sterilized containers and

exposure to air was minimized. Prior to use, the processed core material was maintained under argon and both the core and the water were held at 4°C.

2.3.2 Aerobic Microcosms

Aerobic microcosms were constructed by placing 5 g of crushed core sample and 25 mL of groundwater into 150-mL serum bottles, which were then sealed using Teflon-lined septa and aluminum crimp rings. The appropriate nutrients (see Table 2-1) were added to the groundwater prior to dispensing. In bottles containing methane, the headspace was flushed with a methane/air gas mixture for three minutes prior to sealing. Intrinsic and killed controls were prepared in the same manner. Intrinsic controls consisted of nonamended groundwater and basalt. Killed controls consisted of autoclaved basalt and filter (0.2-μm pore size) sterilized groundwater containing 0.1% sodium azide. The experimental conditions included in the study are listed in Table 2-1. To allow for destructive (sacrificial) sampling, 26 bottles were prepared for each of the active conditions and 14 bottles were set up for each killed control condition.

TCE and *cis*-DCE were added to sealed microcosm bottles as aqueous stock solutions using a syringe pump (Orion Research Inc, Boston, MA) fitted with a 20-mL glass barrel and a 23-gauge needle. After the syringe was loaded, a sample of the stock solution was dispensed and analyzed to determine the chloroethene concentration. The weight of solution added to each bottle was then measured and the concentration of chloroethene in each bottle was calculated. Throughout the study, the bottles were inverted to minimize loss of volatile organics, and incubated statically in the dark at the representative site temperature of 12°C.

2.3.3 Anaerobic Microcosms

Anaerobic microcosms were prepared using the same procedure as the aerobic microcosms. However, groundwater used in these experiments was purged with nitrogen, and bottles were set up in a glove box containing 90% nitrogen and 10% carbon dioxide. In addition, sodium sulfide was added to anaerobic microcosm bottles at a final concentration of 10 mg/L. The experimental conditions included in the anaerobic study are listed in Table 2-2.

2.3.4 Sampling Procedures

Parameters measured over time during aerobic microcosm studies are listed in Table 2-3; those measured over time during anaerobic microcosm and kinetic studies are found in Table 2-4. Initial test conditions were determined by randomly selecting two bottles from each experimental group and analyzing each parameter in duplicate. Sampling was repeated after 2 weeks of incubation and subsequently every 4 to 6 weeks. Once biological activity was observed, microcosms were sampled in triplicate. Aerobic microcosms were measured over a 4-month period and anaerobic microcosms were measured over 7 months.

Table 2-1. Experimental matrix for aerobic microcosm studies.[a]

Treatment Number	Lactate (mM)	Acetate (mM)	Propionate (mM)	Methanol (mM)	Methane (% headspace)	Yeast Extract (mg/L)	$NH_4H_2PO_4$[b] (mM)
1	- IC	-	-	-	-	-	-
2	- KC	-	-	-	-	-	-
3	2.5	5	2.5	-	-	-	-
4	2.5	5	2.5	-	-	50	-
5	2.5	5	2.5	-	-	-	2
6	2.5	5	2.5	-	-	50	2
7	0.5	1	0.5	-	-	50	2
8	-	-	-	15	-	50	-
9	-	-	-	15	-	-	2
10	-	-	-	15	-	50	2
11	0.5	1	0.5	5	-	50	2
12	-	-	-	-	15	50	-
13	-	-	-	-	15	-	2
14	-	-	-	-	15	50	2
15	0.5	1	0.5	-	5	50	2

a. All bottles were spiked with c*is*-DCE at a nominal aqueous level of 5 mg/L

b. Ammonium phosphate

IC = Intrinsic control

KC = Killed control

Table 2-2. Experimental matrix for anaerobic microcosms.

Treatment Number	TCE (mg/L)	cDCE (mg/L)	Lactate (mM)	Propionate (mM)	Methanol (mM)	Yeast Extract (mg/L)	Ammonium Phosphate (mM)	Sodium Sulfate (mM)	Sodium Nitrate (mM)
1	10 KC	-	-	-	-	-	-	-	-
2	10 IC	-	-	-	-	-	-	-	-
3	10	-	2.5	2.5	-	-	-	-	-
4	10	-	2.5	2.5	-	50	-	-	-
5	10	-	2.5	2.5	-	-	5	-	-
6	10	-	2.5	2.5	-	50	5	-	-
7	10	-	2.5	2.5	-	50	5	10	-
8	10	-	2.5	2.5	-	50	5	-	10
9	10	-	0.5	0.5	-	50	5	-	-
10	10	-	0.5	0.5	-	50	5	2	2
11	10	-	-	-	15	50	5	-	-
12	10	-	-	-	15	50	5	10	-
13	10	-	-	-	15	50	5	-	10
14	1 IC	-	-	-	-	-	-	-	-
15	1	-	2.5	2.5	-	50	5	-	-
16	1	-	0.5	0.5	-	50	5	-	-
17	-	5 KC	-	-	-	-	-	-	-
18	-	5 IC	-	-	-	-	-	-	-
19	-	5	2.5	2.5	-	-	-	-	-
20	-	5	2.5	2.5	-	50	5	-	-
21	-	5	2.5	2.5	-	50	5	10	-
22	-	5	2.5	2.5	-	50	5	-	10
23	-	5	0.5	0.5	-	50	5	2	2
24	-	5	-	-	15	50	5	-	-
25	-	5	-	-	15	50	5	2	2

KC =killed control

IC =intrinsic control

Table 2-3. Parameters measured over time during aerobic microcosm studies.

Parameter	Analytical Method	Sample Type	Purpose
cis-DCE	Gas chromatography FID detector	Headspace/SPME	Dehalogenation product
1,1-DCE	Gas chromatography FID detector	Headspace/SPME	Dehalogenation product
VC	Gas chromatography FID detector	Headspace/SPME	Dehalogenation product
Oxygen	Gas chromatography TCD detector	Headspace gas	Electron acceptor
Lactate	Ion chromatography	Aqueous	Added electron donor
Methanol	Gas chromatography FID detector	Aqueous	Added electron donor
Methane	Gas chromatography TCD detector	Headspace gas	Added electron donor
Acetate	Gas chromatography FID detector	Aqueous	Added electron donor
Propionate	Gas chromatography FID detector	Aqueous	Added electron donor

Table 2-4. Parameters measured over time during anaerobic microcosm and kinetic studies.

Parameter	Analytical Method	Sample Type	Purpose
TCE	Gas chromatography FID detector	Headspace/SPME	Contaminant
Cis-DCE	Gas chromatography FID detector	Headspace/SPME	Dehalogenation product
Trans-DCE	Gas chromatography FID detector	Headspace/SPME	Dehalogenation product
1,1-DCE	Gas chromatography FID detector	Headspace/SPME	Dehalogenation product
VC	Gas chromatography FID detector	Headspace/SPME	Dehalogenation product
Ethene	Gas chromatography FID detector	Headspace gas	Dehalogenation product
Ethane	Gas chromatography FID detector	Headspace gas	Dehalogenation product
Methane	Gas chromatography TCD detector	Headspace gas	Anaerobic by-product
Lactate	Ion chromatography	Aqueous	Added electron donor
Methanol	Gas chromatography FID detector	Aqueous	Added electron donor
Propionate	Gas chromatography FID detector	Aqueous	Added electron donor Anaerobic by-product
Acetate	Gas chromatography FID detector	Aqueous	Anaerobic by-product
Butyrate	Gas chromatography FID detector	Aqueous	Anaerobic by-product
Sulfate	Ion chromatography	Aqueous	Electron acceptor
Nitrate	Ion chromatography	Aqueous	Electron acceptor
Nitrite	Ion chromatography	Aqueous	Electron acceptor

3. KINETICS OF ANAEROBIC REDUCTIVE DEHALOGENATION BY INDIGENOUS TAN MICROBES

3.1 Bioreactor Design

The bioreactor used in these studies was a modification of that described by Skeen et al. (1994) and shown in Figure 3-1. The reactor consisted of a 1.2-L stainless steel cylinder measuring 17.5 cm in height and 12.5 cm in diameter. To ensure a gas and watertight seal, the lid and base of the reactor were welded to the cylinder wall. Gas and liquid sample ports, which consisted of 0.6-cm stainless steel plug valves (NUPRO Series 4P), were fitted into the reactor wall. The lid was equipped with two threaded ports, which accepted sterilizable pH and Eh electrodes (Cole-Parmer Co., Vernon Hills, IL). The electrodes were suspended from the reactor lid via stainless steel housings (Cole-Parmer Co., Vernon Hills, IL), which were fitted with Viton O-rings to ensure a tight seal. To monitor leaking, the reactor was equipped with a stainless steel Bourdon tube pressure gauge (Cole-Parmer Co., Vernon Hills, IL), which had a working range of 0 to 30 psig. An Orion 420-A dual-channel pH/mV meter (Orion, Boston, MA) was used to monitor the oxidation-reduction potential and the pH.

3.2 Experimental Set-Up

Fed-batch reactor studies were conducted to determine the kinetics of ARD by indigenous TAN microbes. The experimental conditions used for these studies are listed in Table 3-1. Prior to inoculation, the reactors were autoclaved at 121°C for 20 minutes and cooled to room temperature. They were then loaded using existing TAN cultures isolated during the microcosm study.

Working in a glove box containing nitrogen, anaerobic microcosm cultures, enriched under amendment conditions similar to those chosen for each reactor, were consolidated. The reactor was then loaded with 100 g of recovered basalt, 400 mL of recovered liquid culture, and 400 mL of fresh deoxygenated TAN groundwater. Sodium sulfide was added at a final concentration of 10 mg/L. A stainless steel-encased magnet was added as a stir bar and the reactor was sealed. The addition of liquid (800 mL) and basalt to the reactor resulted in a headspace volume of 0.366 L. Next, using filtered (0.2 μm pore size) nitrogen, the reactor was pressurized to 10 psig and leak tested for 48 hours. Once the reactor passed the leak test, the appropriate amounts of nutrients and chloroethene were added by syringe through the sample ports. In addition to the nutrients listed in Table 3-1, a one time addition of yeast extract (50 mg/L) was provided to the cultures at start up. An abiotic reactor was set up in a similar manner using autoclaved basalt and autoclaved TAN groundwater containing zinc chloride (0.1%).

3.3 Sampling and Analysis

Throughout these studies, the bioreactors were routinely monitored for the parameters listed in Table 2-4. Prior to sampling, pH, Eh, and headspace pressure were recorded. Headspace gases were then analyzed. Next, using a glass syringe (Hamilton Co., Reno, NV), aqueous samples were removed. To minimize basalt loss, the reactor was tilted slightly and the sample withdrawn through the top port. Using this technique, a 5-mL sample was removed and filtered (0.2-μm pore size). The aqueous analytes listed in Table 2-4 were measured in the filtrate. Again using the glass syringe, a 5-mL aqueous sample was removed from the reactor and immediately injected into a sealed 60-mL serum vial. The vial was shaken vigorously for 30 seconds, inverted, and allowed to equilibrate at room temperature for 30 minutes.

Figure 3-1. Bioreactor used in anaerobic kinetic studies.

Table 3-1. Experimental matrix for anaerobic kinetic studies.

Reactor Number	Amendment Conditions							
	TCE (mg/L)	DCE (mg/L)	Lactate (mM)	Glucose (mM)	Methanol (mM)	Sulfate (mg/L)	Nitrate (mg/L)	$NH_4H_2PO_4$[1] (mM)
1	**10**	-	2.5	-	-	Adjust to 40	Adjust to 1	0.5
2	10	-	2.5	-	-	-	-	0.5
3	5	5	2.5	-	-	-	-	0.5
4	10	-	1.5	1.0	5.0	Adjust to 40	Adjust to 1	0.5
5	10	-	1.5	1.0	5.0	-	-	0.5
6	10	-	1.5	1.0	5.0	192	310	0.5
7	10	-		2.0	-	-	-	0.5
8	5	5	5.0	-	-	-	-	0.5
9	5	5		1.5	7.0	-	-	0.5
10	10	Abiotic[2]		-	-	-	-	0.5

[1] Ammonium phosphate.

[2] Abiotic bioreactor consisted of autoclaved basalt and groundwater plus 0.1% zinc chloride.

Chloroethene levels in the headspace of this vial were then measured using the SPME technique and a GC equipped with a FID. Using measured headspace values, the total concentrations of TCE, *cis*-DCE*, trans*-DCE, and 1,1-DCE in the liquid sample were calculated using gas-partitioning constants (Gossett 1987). These numbers in turn were used to calculate the concentration of each chloroethene in the reactor. VC levels were measured in the headspace of each reactor by inserting the SPME fiber through the septum of the gas sample port and extracting for 10 minutes. Liquid picked up on the fiber during the sampling process prevented use of this technique to measure TCE and DCE levels in the reactor headspace.

3.4 Bioreactor Operation

Ten bioreactors were loaded and brought on line using the amendment conditions listed in Table 3-1. Throughout the experiment, the reactors were incubated at 12°C, and mixed using stainless steel-encased magnets and magnetic stir plates. Initially, the reactors were operated under batch conditions and sampled weekly. After 10% of the liquid had been removed from the reactors the levels were readjusted to 800 mL using deoxygenated groundwater. When available carbon was depleted, substrate levels were spiked to initial concentrations. The pH of the cultures was maintained between 7.0 and 8.0 using either sodium hydroxide or phosphoric acid. After approximately 1 month, the reactors were switched to a fed-batch mode. During this phase, the bioreactors were sampled twice weekly and the liquid removed from the reactors was replaced with an equal volume of deoxygenated TAN groundwater containing the appropriate mix and concentration of nutrients.

4. RESULTS AND DISCUSSION

4.1 Degradation Under Aerobic Conditions

Aerobic microcosm studies were conducted to assess the ability of indigenous Well TAN-37 microbes to oxidize chlorinated compounds produced by ARD. At other sites where TCE contamination exists, *cis*-DCE has been observed to be the predominant dehalogenation product (DiStefano et al. 1991, Kastner 1991, and Parsons et al. 1984). Therefore, it was chosen for evaluation in this study. Electron donors used included those potentially available to microbes down gradient from an anaerobic treatment process. To screen for possible nutrient limitations in the field, the effect of yeast extract and ammonium phosphate on the degradation process was assessed. Yeast extract provides bacteria with amino acids, vitamins, and growth factors. Once growth is established, most bacteria can produce these nutrients on their own. Ammonium phosphate was added as a source of both nitrogen and phosphorous. Abiotic controls were included in the study to account for leakage and nonbiological loss of DCE. Intrinsic controls, bottles containing nonamended TAN groundwater and TAN core material, were included to determine if DCE degradation could occur without the addition of an electron donor.

The results of this study are summarized in Table 4-1. Following a 3-month incubation period, *cis*-DCE levels measured in the killed and intrinsic control bottles decreased by approximately 10%. In contrast, complete DCE removal was measured in bottles containing 15% methane and a decrease of 82% occurred in those containing 5% methane. This finding is consistent with earlier studies showing that methanotrophic bacteria capable of oxidizing TCE are indigenous to the TAN aquifer (DOE-ID, 1996). In the previous work, methanotrophic populations were found to be higher near the hot spot, most likely due to elevated levels of nutrients and methane, which were also found in the area. Together these data indicate that if field levels of methane can be increased as a result of an accelerated biotreatment process, methanotrophs will play an active role in degradation of TCE by-products.

Over the course of the experiment, a 64% decrease in DCE was measured in microcosm bottles amended with a mixture of 2.5-mM lactate, 5-mM acetate, and 2.5-mM propionate. These findings demonstrate that these amendments stimulate indigenous cell growth and function as effective co-substrates for oxidative degradation of DCE.

The addition of yeast extract to bottles containing organic acids resulted in a slight increase in DCE removal but did not appear to enhance removal in bottles containing methane. Based upon these findings, the use of yeast extract in the field does not appear to be warranted.

The addition of ammonium phosphate ($NH_4H_2PO_4$) to microcosms containing methane enhanced DCE degradation. However, in bottles containing organic acids, DCE removal appeared to be inhibited. Inhibition was most likely an artifact due to rapid cell growth and subsequent carbon limitation. It could also be that the rapid growth observed was for a population that does not dechlorinate DCE. In bottles containing organic acid plus $NH_4H_2PO_4$, available carbon was depleted within 14 days. In contrast, at the end of the experiment, bottles containing organic acid without $NH_4H_2PO_4$, contained approximately 20 mg/L of acetate, and those amended with $NH_4H_2PO_4$ plus 15% methane contained approximately 6% methane.

Table 4-1. Decrease in *cis*-DCE measured in aerobic microcosm bottles following 4 months of incubation.

% Loss cis-DCE	Lactate (mM)	Acetate (mM)	Propionate (mM)	Methanol (mM)	Methane (%headspace)	Yeast Extract (mg/L)	Ammonium Phosphate (mM)
7	IC -	—	—	—	—	—	—
8	KC -	—	—	—	—	—	—
64	2.5	5	2.5	—	—	—	—
73	2.5	5	2.5	—	—	50	—
25	2.5	5	2.5	—	—	—	2
32	2.5	5	2.5	—	—	50	2
11	0.5	1	0.5	—	—	50	2
24	—	—	—	15	—	50	-
14	—	—	—	15	—	-	2
22	—	—	—	15	—	50	2
15	0.5	1	0.5	5	—	50	2
72	—	—	—	—	15	50	—
100	—	—	—	—	15	—	2
100	—	—	—	—	15	50	2
82	0.5	1	0.5	—	5	50	2

IC = Intrinsic control

KC = Killed control

A similar problem occurred in bottles dosed with methanol. Although low levels of dechlorination were measured, carbon depletion again occurred within the first 2 weeks. Consequently, these experiments did not adequately measure the ability of methanol to stimulate aerobic DCE degradation or provide enough information to fully assess the effect of ammonium phosphate on microbial activity.

4.2 Dechlorination Under Anaerobic Conditions

Dechlorination activity in anaerobic microcosm bottles was not detected until the third month of incubation. At that time, TCE and DCE removal in both the killed control and intrinsic control bottles measured approximately 10%. A loss of 30% TCE was observed in bottles amended with organic acids (lactate plus propionate) and 26% in those containing methanol. Based upon this early data, lactate and methanol were chosen as electron donors for batch reactor studies, and cultures from bottles that had been analyzed were used to inoculate the bioreactors.

The percent chloroethene removal measured in anaerobic microcosms following 7 months of incubation is summarized in Table 4-2.

Table 4-2. Decreases in chloroethene levels measured in anaerobic microcosms following a 7-month incubation period. Experimental matrix treatment numbers are provided in Table 2-2.

Experimental Condition	% Loss of TCE (experimental matrix treatment number)	% Loss of cis-DCE (experimental matrix treatment number)
Killed control	8 (1)	10 (17)
Intrinsic control	15 (2,14)	12 (18)
Lactate + propionate w/ or w/o yeast extract and ammonium phosphate	35 (3, 4, 5, 6, 9, 15, 16)	11 (19, 20)
Above amendment + 10 mM sulfate	14 (7)	15 (21)
Above amendment + 10 mM nitrate	29 (8)	11 (22)
Above amendment + 2 mM sulfate + 2 mM nitrate	26 (10)	10 (23)
Methanol w/ or w/o yeast extract and ammonium phosphate	28 (11)	14 (24)
Above amendment + 10 mM sulfate	11 (12)	NA
Above amendment + 10 mM nitrate	28 (13)	NA
Above amendment + 2 mM sulfate + 2 mM nitrate	NA	10 (25)

NA = Condition + contaminant combination was not evaluated

Throughout the experiment, significant dechlorination was not observed in the intrinsic control bottles. Due to its presence in the TAN groundwater, low levels of acetate (≈ 30 mg/L) were detected at start up. By the end of 7 months, acetate concentrations in the bottles had increased to approximately 250 mg/L. This finding most likely represents microbial degradation of organic matter present in the aquifer materials. The presence of acetate in the groundwater indicates that a similar scenario is occurring in the field. Acetate, however, did not appear to stimulate dechlorination activity.

Over the course of the experiment, DCE degradation did not occur in the anaerobic microcosms, a finding which was reported in other sediment studies (Skeen et al. 1996).

TCE levels measured in anaerobic bottles did not change significantly between 3 and 7 months of incubation. At both sample points, a decrease in TCE of approximately 30% was measured in microcosms amended with a mixture of lactate and propionate. In these bottles, lactate levels were depleted between the second and third month but high levels of propionate and acetate were still present at 7 months. Apparently TCE degradation was stimulated early in the experiment in the presence of lactate. Once lactate was depleted degradation ceased.

In bottles amended with methanol, a decrease of approximately 26% TCE was observed after 3 months of incubation, and a loss of 28% was measured at 7 months. Over the course of the study, methanol levels in the bottles gradually declined but did not become limiting. Although methanol appears to serve as an effective electron donor, it is unclear why higher levels of TCE degradation were not observed over time.

Dechlorination was inhibited by high concentrations of sulfate, presumably due to competition with TCE for electrons. The effect was less pronounced at lower sulfate levels. Under these test conditions, the addition of yeast extract, nitrate, or ammonium phosphate did not appear to enhance TCE degradation.

At the end of 7 months, methane production was not detected indicating the absence of methanogens in the enrichment cultures. Methane has previously been measured in the TAN environment indicating the presence of methanogens in the field. The failure to recover these microbes may be due to the small amount of inoculum used to set up the microcosms, or loss of these microbes during sample collection.

4.3 Bioreactor Studies

The primary focus of the bioreactor studies was to determine the kinetics of ARD by indigenous TAN microbes. In these studies, various combinations of carbon and energy sources were assessed relative to their effect on ARD. The substrates tested included lactate, glucose, and methanol. Lactate and methanol were chosen for study based upon preliminary microcosm data. Glucose was included to assess the feasibility of using a cheap carbon source such as molasses in the field. Chloroethene levels included 5 and 10 mg/L TCE (30.5 and 61 μmol, respectively) and 5 mg/L (41 μmol) *cis*-DCE. In addition, the roles of sulfate and nitrate as potentially competing electron acceptors were evaluated.

4.3.1 Dechlorination Products

In this study, nine bioreactors were inoculated and brought on line. In addition, one reactor was set up and maintained under abiotic conditions to assess leakage and nonbiological loss of TCE. Initially, to allow microbes to acclimate and cell numbers to increase, the reactors were operated under a batch mode. After 32 days of operation, TCE concentrations in several of the reactors began decreasing and low levels of ethene and ethane were detected. After 42 days, headspace levels of ethene and ethane in these reactors had increased dramatically and low levels of *cis*-DCE were observed. These findings demonstrated that indigenous cultures capable of complete dechlorination of TCE had been isolated. At that time, in anticipation of kinetic studies, the reactors were converted to a fed-batch mode and operated at a 53-day hydraulic residence time (HRT). The cultures were allowed to equilibrate under these conditions for approximately 2 months.

During equilibration, TCE concentrations in Reactors 2, 4, 5, 8, and 9 (Table 3-1) continued to decrease and high levels of ethene were measured. TCE degradation data collected from Reactor 2, which was typical of the others, is shown in Figure 4-1. In this graph, the periodic increases in TCE levels correspond to TCE added to the reactor during feeding; subsequent decreases represent degradation. The sharp decrease in ethene and ethane levels measured on days 60 and 82 were due to accidental loss of headspace gas during sampling procedures. Although slight increases in *cis*-DCE were measured, the isomer did not accumulate in the reactor over time. As indicated in Figure 4-2, which shows TCE and ethene/ethane levels in the sterile reactor, abiotic loss of TCE was minimal.

Throughout the first month, redox potentials in all of the reactors ranged between -400 and -500 mV. In the second month, however, the Eh probes began to fail, most likely due to fouling, and the measurements were discontinued. Throughout the experiment, the pH levels in the reactors were maintained between 7.0 and 8.0.

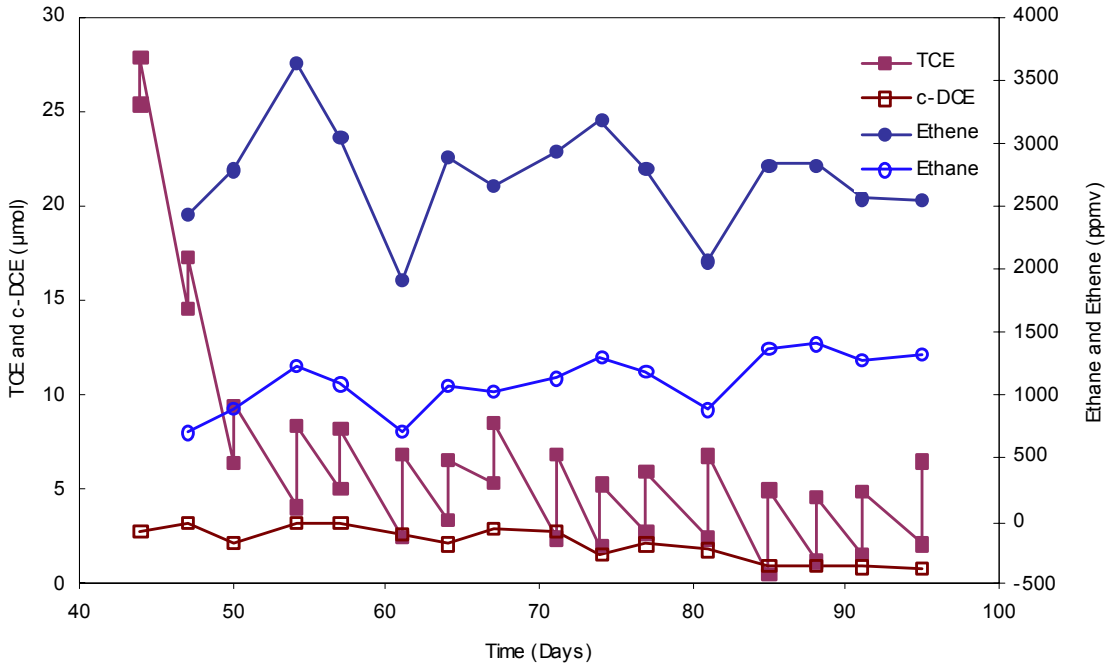

Figure 4-1. TCE degradation by indigenous TAN microbes in fed-batch Reactor 2 receiving lactate and TCE.

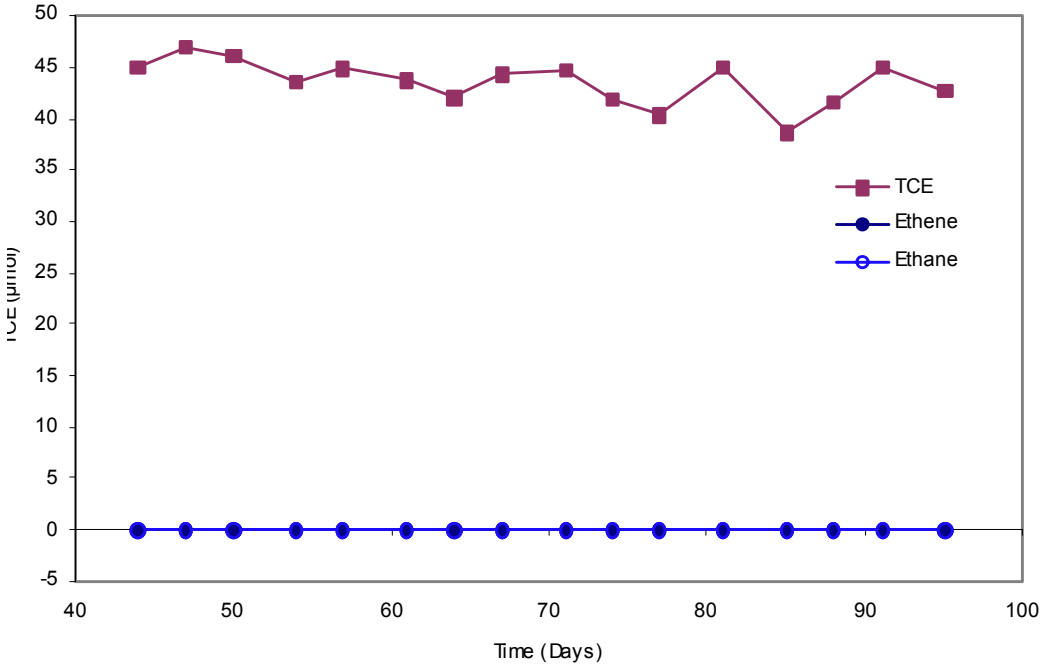

Figure 4-2. TCE and ethene levels in a fed-batch reactor maintained under abiotic conditions.

4.3.2 Relationship Between Dechlorination and Electron Donors and Acceptors

Ethene production in the reactors, which is plotted in Figure 4-3, was used as a qualitative means of assessing the effects of culture amendment on TCE degradation. Over the course of the experiment, ethene was not detected in Reactor 7. This culture was enriched and maintained on glucose. However, high ethene levels were produced in Reactor 9, which received a combination of methanol and glucose. Together these findings indicate that glucose, at least as a sole carbon source, does not stimulate dechlorination activity; whereas, methanol appears well suited for TCE degradation.

Most of the reactors amended with lactate demonstrated significant ARD activity. Inhibition of ARD in Reactor 6, as indicated by low ethene production, can be attributed to high levels of sulfate and nitrate. In anaerobic environments, these anions are commonly used by bacteria as electron acceptors and have been shown to compete with TCE for electrons (Gibson and Suflita 1986). This competitive effect is less pronounced in Reactor 4, which received levels of sulfate and nitrate comparable to the field. It was unclear why ARD activity did not occur in Reactors 1 and 3 which were operated under conditions similar to Reactors 4 and 8, respectively. This may have been due to the presence of different microbial populations in the microcosm bottles that were used to inoculate Reactors 1 and 3.

Methane has previously been measured in TAN groundwater samples indicating the presence of methanogens in the field. However, over the course of these studies, trace levels of methane were detected only in Reactors 4, 5, and 9. As previously mentioned, the failure to recover methanogic bacteria from Well TAN-37 aquifer materials may represent loss of these microbes during sample collection and processing.

4.3.3 Dechlorination Kinetics

4.3.3.1 Kinetic Experiments. After operating the reactors under fed-batch conditions for 2 months, it was apparent that significant ARD activity was occurring in Reactors 2 and 8. Consequently, they were chosen for detailed kinetic studies. In addition, to determine the effects of sulfate and nitrate at levels comparable to the field, Reactor 4 was included. A total of 21 reactor experiments were conducted between March 5 and May 18, 1998. The first-order reaction rate constants derived from these data are summarized in Table 4.3.

In the first set of experiments (March 5, 1998) the reactors were operated at a 53-day HRT, and groundwater containing electron donors and anions at concentrations listed in Table 3-1 was fed into the reactors at a rate of 15 mL/day. TCE and DCE were spiked into the reactors and allowed to equilibrate prior to start up. Initially, samples were taken every 2 hours and analyzed for the parameters listed in Table 2-4. Because very little change in TCE was observed, the sampling schedule was gradually increased to every 12 hours. Throughout the experiment, lactate levels in the reactors fell below detection limit within 4 hours of dosing, however, acetate and propionate, metabolic by-products of lactate, were constantly present.

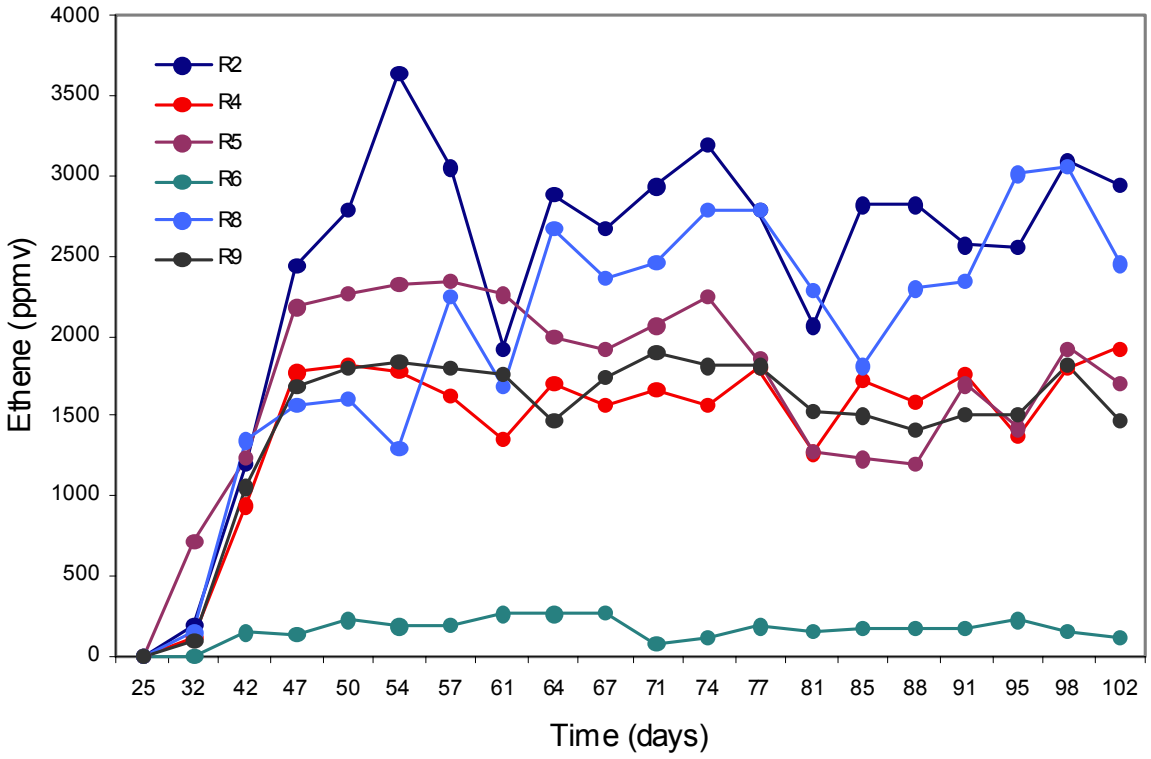

Figure 4-3. Ethene levels in anaerobic fed-batch reactors. Ethene was NOT detected in Reactors 1, 3, 7, and 10.

In subsequent experiments, (March 30, 1998), the HRT was reduced to 20 days, and lactate levels in Reactors 2, 4, and 8 were maintained between 150 and 200 mg/L over a 6-hour period on a daily basis. Concurrently, sulfate levels in Reactor 4 were maintained between 30 and 40 mg/L and nitrate at approximately 1 mg/L. Methanol and glucose that had previously been fed to Reactor 4 were omitted. Samples were collected and analyzed every 2 hours over a period of 6 hours.

During these experiments, gas production and accumulation would be expected as a result of lactate utilization. However, pressure levels in the reactors remained constant or increased only slightly and no apparent loss of headspace gas occurred during sampling. Together these findings indicated that the reactors were slowly leaking. Chloride concentrations in the reactors would also be expected to increase as TCE degradation progressed, but at the present conversion rate, the small increases were difficult to detect due to the presence of high background chloride in the reactors.

In an attempt to resolve the leakage problem, the experiments were modified and repeated. In this set of studies (May 5 and May 18, 1998), the sterile control (Reactor 10) was included and propane was added to the headspace of the reactors as a conservative tracer. Propane is not produced by the bacteria or expected to degrade under anaerobic conditions, thereby providing a means to monitor leakage of ethene and ethane as well as TCE.

4.3.3.2 TCE and DCE Degradation Rate Constants.

Pseudo-first-order rate constants for TCE degradation were successfully estimated for all 21 of the reactor experiments. In addition, pseudo-first-order rate constants for cis-1,2-DCE degradation were estimated for six of the seven experiments conducted in Reactor 8 (which received injections of DCE). Table 4.3 shows estimated pseudo-first-order rate constants (h^{-1} and d^{-1}) and estimated degradation half-lives (d) for TCE and DCE, taking into account gas-liquid mass transfer. Also shown are normalized sum of squared residuals (SSR) values. Normalized SSR, which could also be called normalized mean square error, was calculated by dividing the SSR by the number of data points and by the average compound concentration over the time interval. Dividing by the average compound concentration serves to remove bias associated with large differences in compound concentrations and can be helpful when fitting two curves simultaneously or when comparing curve fits between experiments. First-order model parameters (i.e., initial compound concentration and first-order degradation rate constant) were estimated by fitting model curves to experimental data by time-based, numerical integration. Estimation of individual Michaelis-Menten model parameters was not possible because the parameters correlated with each other mathematically over the range of experimental conditions investigated. Forward point estimates were based on a fourth-order Runge-Kutta method. Optimization was performed to minimize the normalized SSR using the Solver application in Microsoft Excel 97, which is based on a generalized reduced gradient optimization algorithm. To counter difficulties sometimes encountered with numerical procedures and to help Solver identify global, rather than local, minima, the procedure was performed iteratively using different initial parameter values. Available evidence indicates that global minima have been identified in all instances.

As shown in Table 4.3, estimated TCE pseudo-first-order rate constants ranged overall from 0.041 to 0.404 d^{-1}, with average values of 0.132, 0.126, and 0.118 d^{-1} in Reactors 2, 4, and 8, respectively. Based on the first-order model, and accounting for gas-liquid mass transfer in the reactors, these values correspond to estimated TCE degradation half-lives ranging from 2.0 to 19.2 d, with average values of 11.1, 10.4, and 10.1 d in Reactors 2, 4, and 8, respectively. Two experiments conducted in May in Reactor 10, a putative sterile control, showed an average estimated TCE half life of 13.2 d. Estimated DCE pseudo-first-order degradation rate constants for Reactor 8 ranged from 0.063 to 0.707 d^{-1}, with an average value of 0.252 d^{-1}. Again, based on the first-order model, and accounting for gas-liquid mass transfer in the reactor, these values correspond to estimated DCE degradation half-lives ranging from 1.1 to 11.9 d, with an average value of 4.9 d. The fact that DCE accumulations were not observed in Reactors 2 and 4 as TCE degraded is consistent with DCE having a faster degradation rate than TCE.

4.3.3.3 Evaluation of Reactor Leakage with a Propane Tracer.

Experiments were conducted on May 5 and May 18, 1998 in which propane was introduced into the headspace of Reactor 4, 8, and 10 to evaluate leakage of gases and volatile organic compounds. Data from these experiments were modeled using three approaches: (1) as pressure-driven advective transport, (2) as concentration-driven diffusion, and (3) as a first-order loss process. Diffusion coefficients were estimated for propane (382 cm^2/h), TCE (282 cm^2/h), and DCE (318 cm^2/h) using the method of Fuller, Schettler, and Giddings (Lyman et al. 1990). Because the data exhibited significant scatter in two random-looking patterns, one at May 5 and one at May 18, 1998, the overall trend between the two patterns of data could be modeled with the same degree of efficacy using any of the three models. Based on the first-order model, propane disappearance half-lives of 51, 46, and 60 d – considerably longer than estimated TCE and DCE degradation half lives – were estimated for Reactors 4, 8, and 10, respectively. Using the diffusion model and adjusting for differences in propane and TCE diffusion properties, the potential impacts of diffusion losses were evaluated for Reactors 2, 4, and 8, in the March 5, 1998 experiments. These results showed very slight increases in estimated TCE degradation half lives(i.e., from 18.0 to 18.6, from 19.1 to 19.8, and from 16.8 to 17.4 d for Reactors 2, 4, and 8, respectively). Because the effects of leakage on the estimated first-order degradation rate constants appeared to be so minor, and because it is not possible to ascertain at what rate reactor leakage may have developed, no further efforts were made to investigate potential leakage effects.

Table 4-3. Summary of First-Order Reaction Rate Constants.

Experiment	Reactor	k, h^{-1}		k, d^{-1}		Normalized SSR		$t_{1/2}$, d	
Date	No.	TCE	DCE	TCE	DCE	TCE	DCE	TCE	DCE
3/5/98	2	0.00183		0.0440		0.023		18.0	
3/30/98	2 - #1	0.00682		0.1637		0.709		4.8	
3/30/98	2 - #2	0.01459		0.3502		0.018		2.3	
3/30/98	2 - #3	0.00221		0.0531		0.704		14.9	
3/30/98	2 - #4	0.00214		0.0513		0.187		15.4	
High		0.01459		0.3502				18.0	
Low		0.00183		0.0440				2.3	
Mean		0.00552		0.1325				11.1	
S.D.		0.00548		0.1314				7.0	
C.V.		99.2%		99.2%				63.5%	
3/5/98	4	0.00170		0.0408		0.017		19.4	
3/30/98	4 - #1	0.00666		0.1599		0.030		5.0	
3/30/98	4 - #2	0.01682		0.4037		1.480		2.0	
3/30/98	4 - #3	0.00234		0.0562		2.954		14.1	
3/30/98	4 - #4	0.00331		0.0793		0.172		10.0	
5/5/98	4	0.00324		0.0776		0.046		10.2	
5/18/98	4	0.00266		0.0638		0.615		12.4	
High		0.01682		0.4037				19.4	
Low		0.00170		0.0408				2.0	
Mean		0.00525		0.1259				10.4	
S.D.		0.00535		0.1283				5.8	
C.V.		101.9%		101.9%				55.4%	
3/5/98	8	0.00197	0.00261	0.0472	0.0626	0.046	0.037	16.8	11.9
3/30/98	8 - #1	0.00614	0.00827	0.1474	0.1986	0.436	0.345	5.4	3.7
3/30/98	8 - #2	0.01459		0.3501		0.352		2.3	
3/30/98	8 - #3	0.00239	0.02946	0.0574	0.7071	2.122	0.037	13.8	1.1
3/30/98	8 - #4	0.00304	0.00564	0.0730	0.1353	0.198	0.574	10.9	5.5
5/5/98	8	0.00372	0.00851	0.0894	0.2042	0.568	0.136	8.9	3.6
5/18/98	8	0.00255	0.00863	0.0613	0.2071	0.502	0.104	12.9	3.6
High		0.01459	0.02946	0.3501	0.7071			16.8	11.9
Low		0.00197	0.00261	0.0472	0.0626			2.3	1.1
Mean		0.00492	0.01052	0.1180	0.2525			10.1	4.9
S.D.		0.00448	0.00957	0.1076	0.2297			5.0	3.7
C.V.		91.2%	91.0%	91.2%	91.0%			49.8%	75.5%
5/5/98	10	0.00203		0.0487		0.141		16.3	
5/18/98	10	0.00326		0.0783		0.745		10.1	
High		0.00326		0.0783				16.3	
Low		0.00203		0.0487				10.1	
Mean		0.00265		0.0635				13.2	
S.D.		0.00087		0.0210				4.4	
C.V.		33.0%		33.0%				33.0%	

Observation of an estimated TCE half life of 13.2 d in Reactor 10 is perplexing in view of the observed 60-d propane leakage half life in the same reactor over the same time period in May. Because propane is more diffusive than TCE, this observation does not seem explainable in terms of TCE diffusion losses. The reason for this relatively fast TCE disappearance is indeterminate based on currently available data.

4.4 Potential for In Situ Bioremediation of TAN

These studies have shown that indigenous microbes capable of complete ARD are present at Well TAN-37. The kinetic data indicates that controlling the type and concentration of electron donor can increase TCE conversion rates. Although sulfate is known to compete with TCE for electrons, significant dechlorination was observed in studies conducted at sulfate levels comparable to those at TAN. These results suggest that anaerobic activity with similar dechlorination rates may be stimulated in the field. In the event that complete mineralization of TCE does not occur following augmentation, microcosm data indicates that a sequential anaerobic/aerobic treatment scheme is feasible.

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